PYRUVATE KINASE SUBSTRATE ACTIVITY EXHIBITED BY HOMOLOGS OF PHOSPHOENOLPYRUVATE

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SUMMARY

Rabbit muscle pyruvate kinase (EC 2.7.1.40) was found to utilize three homologs of P-enolpyruvate as substrates. These were P-enol-α-ketobutyrate, P-enol-α-ketovalerate and P-enol-α-ketocaproate. The Michaelis constants of these compounds are 2.13 x 10^{-5}M, 1.66 x 10^{-4}M and 1.97 x 10^{-3}M, respectively. The velocity of the enzyme acting on these compounds, relative to P-enolpyruvate was 0.16%, P-enol-α-ketobutyrate; 0.29%, P-enol-α-ketovalerate; 0.13%, P-enol-α-ketocaproate.

Our laboratory recently reported the synthesis and characterization of seven homologs of P-enolpyruvate (1). These included P-enol-α-ketobutyrate, P-enol-α-ketovalerate, P-enol-α- ketocaproate, P-enol-α-ketocaprylate, P-enol-α-ketodecanoate, P-enol-α-ketoisocaproate and P-enol-α-ketoisovalerate. Under the conditions of the assay procedure using approximately 1.0 μg/ml of pyruvate kinase we found these compounds not to act as substrates. However, P-enol-α-ketobutyrate and P-enol-α-ketovalerate were shown to be competitive inhibitors with Ki's of 6.5 x 10^{-5}M and 10.7 x 10^{-4}M, respectively.

More recently it has been reported that P-enol-α-ketobutyrate will serve as a pseudosubstrate with approximately 0.065% of the activity of the reaction relative to P-enolpyruvate (2,3).

In light of these observations, we deemed it advisable to reexamine all of the homologs for possible substrate activity.

MATERIALS AND METHOD

The activity was determined by the method of Pon and Bondar (4) as previously described. The reaction mixtures contained 1.0 mM ADP (Tris

Salt, Sigma Chem. Co.); 10mM Mg^{2+}; 140 mM KCl, 200 μg/ml rabbit muscle pyruvate kinase, and the respective homologs or P-enolpyruvate. The reaction was carried out in 0.05 M Tris-HCl buffer, pH 7.5. Reaction kinetics were determined at 230 nm and 30°C in a Gilford Model 2400 automated spectrophotometer set such that the recorder gave full scale response for 0.1 A. Each point was determined as the initial velocity and also determined at least in duplicate. The Michaelis constants were calculated by the method of least-squares and are the average of multiple determinations.

All of the homologs were rechecked for purity by NMR, IR, U.V., and elemental analysis. P-enol-α-ketobutyrate, P-enol-α-ketovalerate and P-enol-α-ketocaproate were examined by nuclear magnetic resonance for the ratio of cis-trans isomers; i.e., relative to the carboxyl group. They were found to contain respectively 75% cis, 70% cis and 60% cis as indicated by the vinyl proton. The homologs were used as the cyclohexylammonium salts. Melting points discrepencies for P-enol-α-ketobutyrate between Bondinell and Sprinson (2) and Stubbe and Kenyon (3) and Woods, et al. (1) can best be explained by our observations that evolution of gas take place during melting indicating decomposition. The above authors report 154-155°, 147-149°, and 139-140.5° respectively. We have found that the rate at which the temperature of the melting point apparatus increases ultimately determines the melting point. When the temperature is raised slowly, eg., 0.5° per minute a much lower decomposition point is quite sharp. No browning takes place and the decomposition point is quite sharp. On melting, considerable changes were noted in the infrared spectrum of P-enol-α-ketobutyrate which would indicate decomposition.

RESULTS AND DISCUSSION

Three of the homologs exhibited substrate activity; namely, P-enol-α-ketobutyrate, P-enol-α-ketovalerate and P-enol-α-ketocaproate. With enzyme concentrations in excess of 200 μg/ml, no substrate activity

was observed for P-enol-α-ketocaprylate, P-enol-α-ketodecanoate, P-enol-α-ketoisocaproate, P-enol-α-ketoisovalerate, and P-enol-β-phenylpyruvate. Also, when ADP was omitted from the reaction mixture no activity was observed for the three homologs until ADP was added. The Michaelis constants and maximal velocities are shown in Table 1. Also the relative reaction velocities are shown.

Table 1. Substrate Activity of Pyruvate Kinase with Homologs of P-enolpyruvate and their Kinetic Constants:

Substrate	V_{max} (μmoles/ min/mg)	Km(10^5)	Velocity Relative to P-enolpyruvate	Molecular Activity μmoles/ min/μmole enzyme
P-enolpyruvate	110	7.0 ± 0.3[a]	100	26,400
P-enol-α-ketobutyrate	0.172	2.13 ± 0.1[b]	0.16[c]	41.3
P-enol-α-ketovalerate	0.319	16.6 ± 1.0	0.29	76.5
P-enol-α-ketocaproate	0.143	197 ± 8.0	0.13	34.4

[a]Reported by Woods, et al. (1). [b]Stubbe & Kenyon, (3) reported a Km of 2.5 x 10-5M. [c]Stubbe and Kenyon also reported a relative velocity of 0.065 for P-enol-α-ketobutyrate.

It can be observed that the Michaelis constant of P-enol-α-ketobutyrate is in the same order of magnitude, i.e., 10^{-5}M, as that of P-enolpyruvate. We found a Km of 2.13 x 10^{-5}M, for P-enol-α-ketobutyrate which compares favorably to 2.5 x 10^{-5}M reported by Stubbe and Kenyon (3). However, the Km for P-enol-α-ketovalerate was an order of magnitude larger (1.66 x 10^{-4}M) and for P-enol-α-ketocaproate another order of magnitude (1.97 x 10^{-3}M).

In Table 1 are also shown the molecular activities for pyruvate kinase acting on each of the substrates. These striking differences point out the very slow activity of pyruvate kinase toward the homologs compared to P-enolpyruvate. As the alkyl group attached to the C-3

position becomes larger the Michaelis constant decreases rapidly as shown in Table 1 indicating marked decrease in the affinity of the enzyme for the substrate. This supports our contention that the bulkiness of the alkyl groups decrease the affinity which was also shown by the K_i values originally reported (1).

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